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Novel antibody-based drugs for PD-L1 and TRAIL-R targeted cancer immunotherapy

Hendriks, Djoke

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CHAPTER 4

Programmed Death Ligand 1 (PD-L1)-targeted TRAIL combines PD-L1-mediated checkpoint inhibition with TRAIL-mediated apoptosis induction

Djoke Hendriks[1,*], Yuan He[1,*], Iris Koopmans[1], Valerie R. Wiersma[1], Robert J. van Ginkel[1], Douwe F. Samplonius[1], Wijnand Helfrich[1] and Edwin Bremer[2,3]

[1]University of Groningen, University Medical Center Groningen (UMCG), Department of Surgery, Laboratory for Translational Surgical Oncology, Groningen, The Netherlands. [2]UMCG, Department of Hematology, section Immunohematology. [3]University of Exeter Medical School, St Luke's Campus, Exeter, Devon, UK.

* Authors contributed equally

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Abstract

Antibodies that block PD-L1/PD-1 immune checkpoints restore the activity of functionally-impaired antitumor T cells. These antibodies show unprecedented clinical benefit in various advanced cancers, particularly in melanoma. However, only a subset of cancer patients responds to current PD-L1/PD-1-blocking strategies, highlighting the need for further advancements in PD-L1/PD-1-based immunotherapy. Here, we report on a novel approach designed to combine PD-L1 checkpoint inhibition with the tumor-selective induction of apoptosis by TNF-related Apoptosis Inducing Ligand (TRAIL). In brief, a new bi-functional fusion protein, designated anti-PD-L1:TRAIL, was constructed comprising a PD-L1 blocking antibody fragment genetically fused to the extracellular domain of the pro-apoptotic tumoricidal protein TRAIL. Treatment of PD-L1-expressing cancer cells with anti-PD-L1:TRAIL induced PD-L1-directed TRAIL-mediated cancer cell death. Treatment of T cells with anti-PD-L1:TRAIL augmented T cell activation, as evidenced by increased proliferation, secretion of IFN-γ and enhanced killing of cancer cell lines and primary patient-derived cancer cells in mixed T cell/cancer cell culture experiments. Of note, elevated levels of IFN-γ further up-regulated PD-L1 on cancer cells and simultaneously sensitized cancer cells to TRAIL-mediated apoptosis by anti-PD-L1:TRAIL. Additionally, anti-PD-L1:TRAIL converted immunosuppressive PD-L1-expressing myeloid cells into pro-apoptotic effector cells that triggered TRAIL-mediated cancer cell death. In conclusion, combining PD-L1 checkpoint inhibition with TRAIL-mediated induction of apoptosis using anti-PD-L1:TRAIL yields promising multi-fold and mutually reinforcing anticancer activity that may be exploited to enhance the efficacy of therapeutic PD-L1/PD-1 checkpoint inhibition.

Introduction

Programmed Death Ligand 1 (PD-L1) and its cognate receptor PD-1 represent an immune checkpoint of great interest for cancer immunotherapy. Antibodies that block PD-L1/PD-1 interaction restore the anticancer activity of functionally impaired tumor infiltrating lymphocytes (TILs), specifically cytotoxic T cells. Treatment with these antibodies has transformed the landscape of cancer immunotherapy, yielding long-term remission and cure in a subset of advanced stage melanoma patients.[1, 2]

The PD-L1/PD-1 immune checkpoint normally ensures timely shut-down of immune responses to prevent collateral damage or autoimmunity (reviewed in [3]). In brief, PD-L1 expression is upregulated on antigen-presenting cells during inflammation by locally produced IFN-γ and is expressed by myeloid suppressor cells.[4-6] Simultaneously, expression of PD-1 increases on activated T cells[7], which upon interaction with PD-L1 dampens the cytolytic activity of T cells[4]. Interestingly, activated T cells not only express PD-1, but upon activation also upregulate PD-L1.[8, 9] Antibody-mediated cross-linking of PD-L1 on T cells triggers co-stimulatory signaling and ultimately leads to induction of apoptosis[10], indicative of an immunoregulatory role for PD-L1 on T cells.

Various cancer types upregulate PD-L1 expression either constitutively via oncogenic signaling pathways or in response to IFN-γ produced in the tumor environment.[11, 12] Consequently, antitumor T cells are inhibited via PD-L1/PD-1 interaction, which allows cancer cells to evade the immune system even in highly immunogenic malignancies such as melanoma.[12, 13] Hence, the expression of PD-L1 on cancer cells often correlates with unfavorable prognosis.[14, 15] Although PD-1 and PD-L1-blocking antibodies have triggered breakthrough curative anticancer immunity, most notably in advanced melanoma,[1, 2] the benefit of these antibodies is still restricted to a minority of cancer patients.

To expand the clinical effects of immune checkpoint therapy, various combinatorial approaches have been attempted in order to identify opportunities for synergistic activity. For instance, treatment with PD-1-blocking antibody nivolumab was combined with CTLA-4 antibody ipilimumab, which significantly enhanced response rates in melanoma patients.[16] Similarly, combination of PD-1/PD-L1 blockade with induction of cytotoxic cancer cell death by radiotherapy proved more effective and enhanced activation of anticancer immunity.[17, 18] These selected examples highlight that more efficacious PD-L1/PD-1 targeted therapy can be achieved by rational design of combinatorial therapeutic approaches.

In this respect, we and others have previously reported on a class of bi-functional fusion proteins that comprise an scFv antibody fragment genetically fused to the tumoricidal protein tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) (reviewed in [19]). TRAIL is a homotrimeric death-inducing ligand of the TNF superfamily with well-documented tumor-selective pro-apoptotic activity that has been proven safe in clinical trials (reviewed in [20]). Antibody fragment-mediated delivery of soluble TRAIL optimizes its target cell-selective accretion and, moreover, triggers enhanced TRAIL-receptor mediated apoptosis in targeted cancer cells.[21, 22] Importantly, use of an scFv antibody fragment with antagonistic activity equips the scFv:TRAIL fusion protein with addi-

tional tumoricidal activity, e.g. by inhibition of EGFR-mitogenic signaling[23] or by blocking tumor-expressed CD47 and thereby augmenting neutrophil-mediated phagocytosis of cancer cells[24]. Furthermore, scFv-mediated display of TRAIL on the surface of T cells or granulocytes augments the cytolytic activity of these immune effector cells.[25, 26]
Based on this bi-functional TRAIL-based fusion protein format, we constructed and pre-clinically evaluated an anti-PD-L1:TRAIL fusion protein comprised of a PD-L1 blocking antibody fragment genetically fused to human soluble TRAIL. This anti-PD-L1:TRAIL fusion protein was designed to combine PD-L1 checkpoint inhibition with simultaneous TRAIL-mediated activation of cancer cell death.

Materials & methods

Antibodies, Reagents, Inhibitors

The following antibodies were used in this study; anti-CD279-PE (PD-1, clone MIH4, eBioscience), anti-CD274-APC (PD-L1, clone 29E.2A3, BioLegend), anti-CD83-PE (clone HB15e, eBioscience), anti-CD206-PE (clone 19.2, eBioscience), anti-CD3-PerCP-Cyanine5.5 (clone OKT-3, eBioscience), anti-TRAIL/TNFSF10-PE (clone 75402, R&D systems), NG2-FITC (anti-MCSP, clone LHM2, Santa Cruz Biotechnology), polyclonal Goat-anti-Human-PE (SouthernBiotech), Goat-anti-Mouse IgG (H+L) Secondary Antibody (Alexa Fluor 488 conjugate, Thermo Scientific) and anti-CD4-APC (clone MEM-241), anti-CD8-FITC (clone HIT8a), anti-CD56-PE (clone B-A19), anti-CD14-FITC (clone MEM-15), anti-CD11b-FITC (clone MEM-174), anti-HLA-DR-PE (clone MEM-12), anti-CD86-FITC (clone BU63), Mouse IgG2b-APC, Annexin-V-FITC (all Immunotools). Recombinant human IFN-γ, GM-CSF, M-CSF, IL-4, IL-10, TGF-β1, anti-CD3 (clone UCHT-1) and anti-IFN-γ (clone B27) were purchased from ImmunoTools. LPS (Lipopolysaccharides from E. coli 0111:B4) was purchased from Sigma-Aldrich. Recombinant human PD-1:Fc was purchased from R&D systems. Pan-caspase inhibitor z-VAD-fmk, TRAILR1 (clone DJR1) and TRAILR2 (clone DJR2-4) antibodies were purchased from Enzo Life Sciences. TRAIL-neutralizing mAb 2E5 was purchased from Life Technologies. Recombinant CMV protein pp65 was purchased from Miltenyi Biotec. A PD-L1 neutralizing murine antibody was purchased from BPS Bioscience.

Cell Lines

DLD-1, HCT-116, SK-MEL-28, A2058 and CHO-K1, NCI-H1975, ES-2, MDA-MB-231 were obtained from the American Type Culture Collection (ATCC). TRAIL-resistant cell line HCT-116.cFLIPs was kindly provided by Prof. dr. Harald Wajant (University of Würzburg, Würzburg, Germany). All cell lines were cultured in RPMI-1640 or DMEM (Lonza) supplemented with 10% fetal calf serum (FCS, Thermo Scientific). DLD-1.PD-L1 cells were generated by transfection of parental DLD-1 cells with eukaryotic expression plasmid pCMV6-PD-L1 using Fugene-HD (Promega). Stable transfectants were generated using Hygromycin B selection (Life technologies). All cells were cultured at 37°C, in a humidified 5% CO_2 atmosphere. Cell numbers were quantified using a cell counter (Sysmex). For experiments, tumor cells were cultured in 48-wells plates at a density of $2x10^4$ cells/well.

For up-regulation of PD-L1, cells were pre-treated for 24 hours with 20 ng/ml IFN-γ. PD-L1 expression was analyzed with an Accuri C6 flow cytometer (BD Biosciences) using PD-L1-APC antibody or appropriate isotype control. Relative PD-L1 expression levels are listed in Supplementary Table 1. TRAIL receptor expression was determined by flow cytometry using TRAILR1 and TRAILR2 antibodies with secondary Goat-anti-Mouse-488 conjugate staining. Relative TRAIL receptor expression levels are listed in Supplementary Table 2.

Primary patient-derived melanoma cells and Tumor Infiltrating Lymphocytes

Fresh melanoma and appendix carcinoma tissue was collected during surgical resection after informed consent (local approval nr. METc2012/330). Tissue was minced and cultured in RPMI 1640 with 10% FCS. Adherent cell phenotype was analyzed by flow cytometry using fluorescently labeled CD14, PD-L1 and MCSP antibodies. Primary patient-derived melanoma cells used in this study were CD14-negative and MCSP-positive and were used before passage 4. For generation of Tumor Infiltrating Lymphocytes (TILs), minced tissue fragments were cultured in RPMI 1640 with 10% FCS supplemented with 50 IU/ml IL-2 (Proleukin, Novartis). TIL phenotype was analyzed by flow cytometry for CD3, CD4, CD8 and CD56.

Production of TRAIL fusion proteins

Anti-PD-L1:TRAIL was constructed by insertion of an anti-PD-L1 mAb 3G10-derived scFv into Sfi1 and Not1 restriction sites into the previously described plasmid pEE14-scFv:TRAIL.[27] Briefly, CHO-K1 cells were transfected with eukaryotic expression plasmid pEE14scFv:sTRAIL using the Fugene-HD reagent (Promega) and stable transfectants were generated by the glutamine synthetase selection method. Stable transfectants were cultured at 37°C in serum-free CHO-S SFM II suspension medium (Gibco, Life Technologies) for up to 7 days after which supernatant was harvested (1,500g, 10 min) and stored at −20°C until further use. Fusion protein concentration in culture supernatant was determined by TRAIL ELISA (Abcam). Anti-EpCAM:TRAIL and anti-MCSP:TRAIL were described before.[22, 27]

PD-L1 specific binding of anti-PD-L1:TRAIL

Tumor cells were incubated with anti-PD-L1:TRAIL (1 μg/ml) for 1 hour at 4°C, washed twice with PBS (1000g, 5 min), stained with anti-TRAIL-PE for 30 minutes at 4°C, washed twice with PBS, and analyzed for binding by flow cytometry. Where indicated tumor cells were pre-incubated with excess (10 μg/ml) PD-L1 blocking mAb.

PD-1/PD-L1 blocking by anti-PD-L1:TRAIL

DLD-1 and DLD-1.PD-L1 cells were pre-incubated with indicated concentrations of anti-PD-L1:TRAIL for 1 hour at 0ºC, after which cells were washed twice (1000g, 5 min) and incubated with 4 μg/ml PD-1:Fc for 1 hour at 0ºC. Subsequently, cells were washed twice (1000g, 5 min) and stained with Goat-anti-Human-PE for 30 min at 0°C and washed twice (1000g 5 min). PD-1:Fc binding was evaluated by flow cytometry.

Apoptosis assay

Tumor cells were treated with anti-PD-L1:TRAIL or anti-EpCAM:TRAIL and, where indicated, in the presence of PD-L1 blocking mAb (10 µg/ml), pan-caspase inhibitor z-VAD-FMK (10 µM) or TRAIL-neutralizing mAb 2E5 (1 µg/ml). After 18 hours, apoptosis was assessed by flow cytometry using Annexin-V staining according to manufacturer's protocol (Immunotools). Where indicated, cells were co-treated with 1 µg/ml cycloheximide (CHX, Sigma-Aldrich).

Spheroid assay

DLD-1, DLD-1.PD-L1 or NCI-H1975 cells (1-5x10^3 cells/well) were seeded in low adherence 96-well plates (Costar) in DMEM supplemented with MITO+ Serum Extender (Corning). Cells were treated with anti-PD-L1:TRAIL, anti-EpCAM:TRAIL or anti-MCSP:TRAIL as indicated. After 72 hours, cell viability was determined using MTS (CellTiter 96 AQueous One Solution Cell Proliferation, Promega) at 490 nM using a Victor V3 multi-label plate counter (Perkin Elmer). Absorbance of the maximum death control (treatment with 70% ethanol for 15 min) was subtracted from all values, after which cell viability was calculated as percentage of medium control. Light microscopy images were acquired at 10x magnification using the EVOS XL core cell imaging system (Life Technologies) and colony number was counted manually in three separate fields-of-view per condition in triplicates. Pre-formed spheroids of DLD-1 and DLD-1.PD-L1 cells were generated by 96 hours culture in low adherence flasks (Costar), after which spheroids were transferred to low adherence plates for experiments.

PBMC stimulation assays

Peripheral blood mononuclear cells (PBMCs) were obtained from venous blood of healthy volunteers after informed written consent using standard density gradient centrifugation (Lymphoprep). PBMCs (1.25x10^5/well) were cultured in a 48-well plate in the presence of 0.5 µg/ml agonistic CD3 mAb (UCHT-1) and indicated concentrations anti-PD-L1:TRAIL, anti-EpCAM:TRAIL or PD-L1 mAb. After 72 hours, total cell number was quantified using an automated cell counter (Sysmex) and culture supernatants were stored at -20°C. IFN-γ levels in culture supernatant were determined by IFN-γ ELISA (eBioscience). Where indicated, freshly isolated PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE) (CellTrace CFSE Cell Proliferation Kit, Invitrogen), and after 72 hours of respective treatment the cell proliferation was analyzed by flow cytometry within the live PBMCs (defined by FSC/SSC gating).

For CMV specific responses, freshly isolated PBMCs from CMV negative and positive donors were cultured in 96-well plates (1.5x10^5/well) in the presence of CMV pp65 according to manufacturer's instructions (Miltenyi Biotec). After 96 hours, culture supernatants were stored at -20°C and secreted IFN-γ was determined by IFN-γ ELISA.

Antitumor reactivity assay

Tumor cells were labeled with DiD (Vybrant Cell-Labeling Solution, LifeTechnologies). Subsequently, 2x10^4 tumor cells were co-cultured with freshly isolated PBMCs or $CD3^+$ T cells (98% purity, using the human Pan T Cell Isolation Kit (Miltenyi Biotec)) in the presence of 0.5 µg/ml agonistic CD3 mAb (UCHT-1) or CD3/CD28 beads (Dynabeads Human T-Activator CD3/CD28, Thermo Fischer) at a bead-to-cell ratio of 1:10, respectively. Mixed cultures were further treated with 0.5 µg/ml anti-PD-L1:TRAIL, anti-EpCAM:TRAIL or PD-L1 mAb. After 48 hours, loss of mitochondrial membrane potential (Δψ) in DiD labeled tumor cells was analyzed by DiOC6 staining (Eugene) as previously described.[27] After harvesting, PBMCs were stained with fluorescent CD4 and CD8 antibodies whereupon the number of $CD4^+$ and $CD8^+$ T cells within the PBMC gate was analyzed by flow cytometry.

Alternatively, 5x10^4 DLD-1 cells were pre-seeded 24 hours before addition of freshly isolated PBMCs. Tumor cells were mixed with PBMCs at indicated E:T ratio's and co-treated with 50 ng/ml BIS-1 (anti-EpCAM:anti-CD3 bispecific antibody previously described in[28]) and 0.5 µg/ml anti-PD-L1:TRAIL or anti-MCSP:TRAIL. After 24 hours, non-adherent cells were carefully washed away and cell viability was determined using MTS as described above.

Patient-derived melanoma and appendix carcinoma cells were co-cultured with autologous TILs in E:T ratio of 2:1. After 48 hours, apoptosis was assessed by Annexin-V staining and IFN-γ levels in culture supernatant were determined by ELISA.

Isolation and generation of myeloid-derived cell types

Monocytes: PBMCs were subjected to magnetic-activated cell sorting (MACS) with anti-CD14-beads and MS columns (Miltenyi Biotec). ***Macrophages:*** monocytes (1x10^6/ml) were treated with 50 ng/ml M-CSF for 6 days, yielding M0 macrophages. M0 macrophages were subsequently stimulated with 50 ng/ml LPS and 20 ng/ml IFN-γ to generate M1 macrophages or 20 ng/ml IL-4, IL-10 and TGF-β1 to generate M2 macrophages. Macrophage phenotype was confirmed by flow cytometric analysis of CD14, CD206 and PD-L1, as described before.[29] ***Dendritic cells:*** immature DCs (iDCs) were generated by treatment of monocytes (3x10^6/ml) with 500 U/ml GM-CSF and 1000 U/ml IL-4 for 7 days. Mature DCs (mDCs) were generated by treatment of iDCs with 1 µg/ml LPS for 3 days. DC phenotype was confirmed by flow cytometric analysis of PD-L1, CD83, CD14, HLA-DR and CD86.

For mixed culture experiments, myeloid cell types were mixed with 2.5x10^3 DLD-1 cells (at E:T ratio 4:1) with the indicated concentrations of anti-PD-L1:TRAIL in presence or absence of PD-L1 mAb. After 18 hours, DLD-1 cells were analyzed for apoptosis by flow cytometric analysis of Annexin-V staining. DLD-1 cells were separately analyzed by excluding $CD14^+$ (monocytes and macrophages) or $CD11b^+$ (DCs) cells.

Statistical analysis

Statistical analysis was performed by two-way ANOVA followed by Tukey-Kramer post-test, one-way ANOVA followed by Tukey-Kramer post-test, Wilcoxon matched pairs test or two-sided unpaired Student t test as indicated using Prism software. $P<0.05$ was defined as a statistically significant difference. Where indicated * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$.

Results

anti-PD-L1:TRAIL induces PD-L1 restricted TRAIL-mediated apoptosis in cancer cells

The anti-PD-L1:TRAIL fusion protein was designed to bind to PD-L1 on cancer cells and subsequently trigger TRAIL-mediated apoptosis by activating agonistic TRAIL-receptors. In line with this, anti-PD-L1:TRAIL strongly and dose-dependently bound to carcinoma cell line DLD-1.PD-L1 that ectopically overexpress PD-L1 (Fig. 1A). In contrast, anti-PD-L1:TRAIL did not bind to parental DLD-1 cells (Fig. 1B and Suppl. Fig. 1A). Binding of anti-PD-L1:TRAIL to DLD-1.PD-L1 was abrogated by co-incubation with molar excess of epitope-competing anti-PD-L1 monoclonal antibody (Fig. 1A). Treatment of DLD-1.PD-L1 with anti-PD-L1:TRAIL triggered dose-dependent TRAIL-mediated apoptosis, whereas similar treatment of DLD-1 cells did not trigger apoptosis (Fig. 1C). Thus, anti-PD-L1:TRAIL triggers cell death specifically after binding to cell surface-expressed PD-L1. Of note, treatment of DLD1.PD-L1 with PD-L1-blocking antibody alone and anti-EpCAM:TRAIL alone induced ~20% and 45% apoptosis, respectively (Fig. 1D). Combined treatment with PD-L1-blocking antibody and anti-EpCAM:TRAIL additively enhanced apoptosis to ~65% (Fig. 1D). However, treatment with anti-PD-L1:TRAIL at the same concentration induced up to 90% apoptosis (Fig. 1D). The apoptotic activity of anti-PD-L1:TRAIL was abrogated when cells were treated in the presence of excess epitope-competing PD-L1 monoclonal antibody (Fig. 1E). Further, apoptotic activity was TRAIL-mediated since TRAIL-neutralizing monoclonal antibody or total caspase-inhibitor z-VAD-fmk blocked apoptosis induction in DLD-1.PD-L1 (Fig. 1E). Of note, DLD-1 and DLD.PD-L1 are equally sensitive to TRAIL-mediated apoptosis since control EpCAM-targeted anti-EpCAM:TRAIL, that binds equally well to both cell lines (data not shown), induced apoptosis to a similar extent in both cell lines (Fig. 1D). In contrast, a control non-targeted fusion protein, anti-MCSP:TRAIL, only minimally induces apoptosis in either cell line (Fig. 1E). In a small panel of cancer cell lines that naturally express PD-L1 (Suppl. Table 1), treatment with anti-PD-L1:TRAIL also induced apoptosis (Fig. 1F). Anti-PD-L1:TRAIL treatment further abrogated spheroid formation in DLD-1.PD-L1, but not DLD-1 cells (Fig. 1G-H), and strongly reduced viability of established spheroids (Fig. 1I). In spheroid forming assays, anti-PD-L1:TRAIL also significantly reduced cell viability of NCI-H1975 cells that endogenously express PD-L1 (Suppl. Fig 1B-C). Thus, apoptotic activity of anti-PD-L1:TRAIL is dependent on PD-L1 specific binding to target cells and subsequent induction of TRAIL-mediated apoptosis.

anti-PD-L1:TRAIL blocks PD-1/PD-L1 interaction and enhances T cell activation

Since TRAIL is a naturally occurring homotrimer the anti-PD-L1:TRAIL fusion protein contains three PD-L1 blocking scFvs, which should allow for effective inhibition of PD-L1/PD-1 interaction. Indeed, anti-PD-L1:TRAIL dose-dependently inhibited binding of recombinant PD-1:Fc to DLD-1.PD-L1 cells, whereas similar treatment with anti-EpCAM:TRAIL did not affect PD-1:Fc binding (Fig. 2A). In line with the PD-L1/PD-1 blocking effect of anti-PD-L1:TRAIL, treatment of peripheral blood mononuclear cells (PBMCs) with anti-PD-L1:TRAIL and an agonistic CD3 mAb increased proliferation and cell number 2-fold

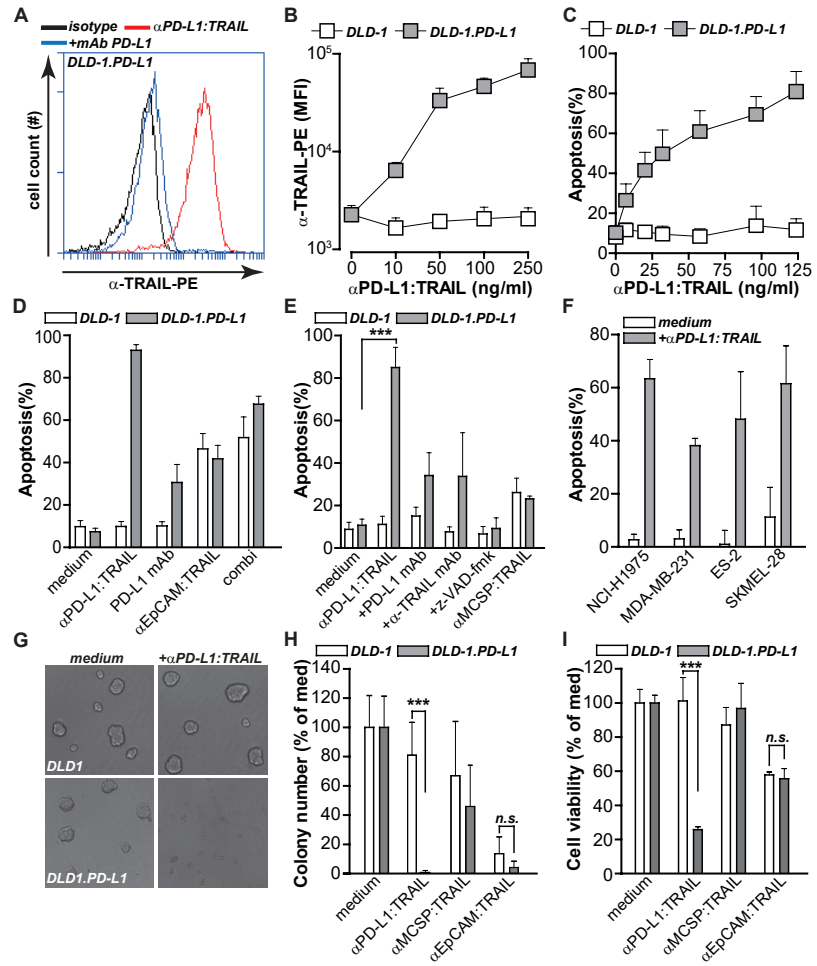

Figure 1: anti-PD-L1:TRAIL induces PD-L1-restricted TRAIL-mediated apoptosis in cancer cells. **A)** Binding of anti-PD-L1:TRAIL to DLD-1.PD-L1 cells in the presence or absence of excess PD-L1 blocking antibody (10 μg/ml) was analyzed by flow cytometry. **B)** DLD-1.PD-L1 or DLD-1 cells were incubated with an increasing dose of anti-PD-L1:TRAIL and binding was assessed by flow cytometry. **C)** DLD-1.PD-L1 or DLD-1 cells were treated with an increasing dose of anti-PD-L1:TRAIL for 18 hours, after which apoptosis was measured by flow cytometry using Annexin-V staining. **D)** DLD-1.PD-L1 or DLD-1 cells were treated with anti-PD-L1:TRAIL (250 ng/ml), anti-EpCAM:TRAIL (250 ng/ml) or PD-L1 antibody (1 μg/ml). Apoptosis was assessed by Annexin-V staining after 18 hours. **E)** DLD-1.PD-L1 or DLD-1 cells were treated with anti-PD-L1:TRAIL (250 ng/ml) in the presence or absence of PD-L1 blocking antibody (10 μg/ml), TRAIL-neutralizing mAb (1 μg/ml) or total caspase inhibitor z-VAD-fmk (10 μM). DLD-1.PD-L1 or DLD-1 cells were also treated with anti-MCSP:TRAIL(250 ng/ml). Apoptosis was assessed by Annexin-V staining after 18 hours. **F)** PD-L1-expressing cell lines were co-treated with cycloheximine (CHX, 1 μg/ml) and anti-PD-L1:TRAIL (1 μg/ml). Apoptosis was determined by Annexin-V staining after 18 hours. **G)** Representative light microscopy images of spheroid size of DLD-1.PD-L1 cells or DLD-1 cells in medium control versus anti-PD-L1:TRAIL-treated conditions after 72 hours. **H)** Spheroid formation of DLD-1.PD-L1 or DLD-1 cells was assessed in the presence or absence of 100 ng/ml anti-PD-L1:TRAIL, anti-MCSP:TRAIL or anti-EpCAM:TRAIL. Number of spheroid colonies was determined after 72 hours by counting three fields-of-view per condition in triplicates. **I)** Established spheroids of DLD-1.PD-L1 cells or DLD-1 cells were treated with 500 ng/ml anti-PD-L1:TRAIL, anti-MCSP:TRAIL or anti-EpCAM:TRAIL. Cell viability was determined by MTS after 72 hours. All graphs represent mean±SD. Statistical analysis was performed using two-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001, n.s. not significant).

compared to treatment with the agonistic CD3 mAb alone (Fig. 2B, C). Furthermore, anti-PD-L1:TRAIL dose-dependently increased secretion of IFN-γ (Fig. 2D), to a level similar to that induced by a monoclonal antagonistic anti-PD-L1 antibody (Fig. 2E). This T cell stimulatory effect of anti-PD-L1:TRAIL was also detected in mixed cultures of PBMCs with DLD-1 cells (Suppl. Fig. 1D). Importantly, co-treatment with anti-EpCAM:TRAIL did not increase T cell proliferation or T cell number compared to CD3 activation alone (Fig. 2B, C). To subsequently investigate pro-inflammatory activity of anti-PD-L1:TRAIL in an antigen-specific reaction, a model system using Cytomegalovirus (CMV)-specific T cells was used. To this end, PBMCs from CMV^{pos} and CMV^{neg} donors were loaded with CMV protein pp65 in combination with anti-PD-L1:TRAIL or anti-EpCAM:TRAIL. Loading of PBMCs with pp65 in the presence of anti-PD-L1:TRAIL significantly increased IFN-γ secretion by CMV^{pos} donor cells (Fig. 2F), whereas no effect on IFN-γ secretion was detected in T cells from CMV^{neg} donors.

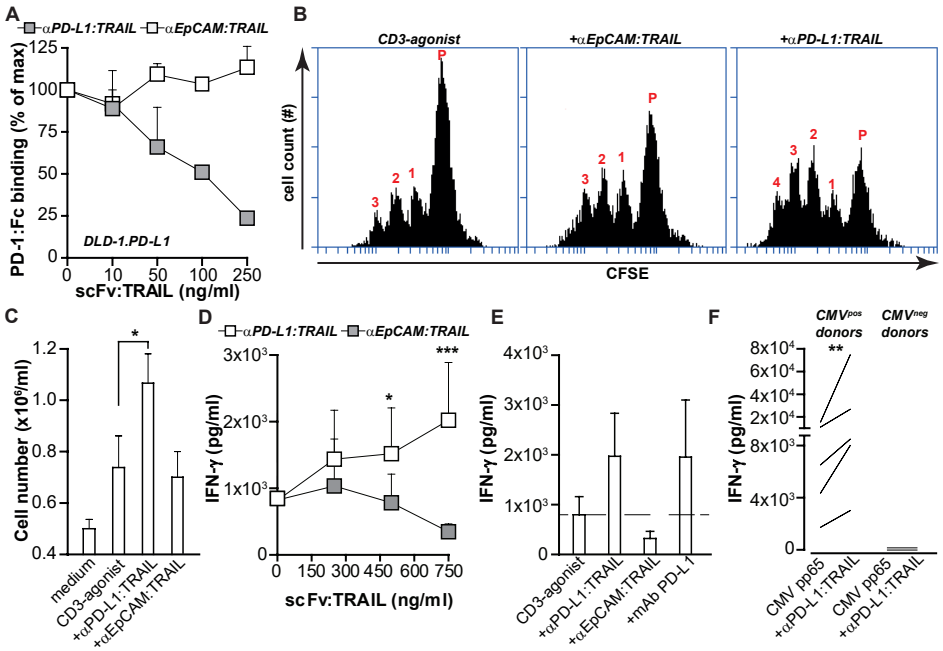

Figure 2: anti-PD-L1:TRAIL blocks the PD-1/PD-L1 interaction and enhances T cell activation. **A)** Binding of PD-1:Fc (4 µg/ml) to DLD-1.PD-L1 cells in the presence of an increasing dose of anti-PD:L1:TRAIL or anti-EpCAM-TRAIL was analyzed by flow cytometry. **B)** Representative histograms of CFSE-labeled PBMCs co-treated with agonistic CD3 mAb (0.5 µg/ml) and 500 ng/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL. After 72 hours, cell proliferation was analyzed by flow cytometry. **C)** PBMCs were treated with 500 ng/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL in the presence of agonistic CD3 mAb (0.5 µg/ml). After 72 hours, cell number was quantified using an automated cell counter. **D)** PBMCs were co-treated with agonistic CD3 mAb (0.5 µg/ml) and an increasing dose of anti-PD-L1:TRAIL or anti-EpCAM:TRAIL. After 72 hours, IFN-γ levels in culture supernatant were determined by ELISA. **E)** PBMCs were co-treated with agonistic CD3 mAb (0.5 µg/ml) and 500 ng/ml anti-PDL1:TRAIL, anti-EPCAM:TRAIL or mAb PD-L1 for 72 hours. IFN-γ levels in culture supernatant were determined by ELISA. **F)** PBMCs from CMV-positive or CMV-negative donors and were treated with 500 ng/ml anti-PD-L1:TRAIL in the presence of CMV protein pp65 for 96 hours. IFN-γ levels in culture supernatant were determined by ELISA. All graphs represent mean±SD. Statistical analysis was performed using unpaired two-sided Student t test (C), two-way ANOVA (D) or Wilcoxon matched pairs test (F) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant).

Importantly, anti-EpCAM:TRAIL only minimally induces IFN-γ secretion (Suppl. Fig. 1E). Taken together, anti-PD-L1:TRAIL potentiates T cell proliferation and IFN-γ production via blockade of PD-1/PD-L1 interaction.

PD-L1:TRAIL enhances anticancer T cell activity

Next, we analyzed whether enhanced T cell activation by anti-PD-L1:TRAIL could augment anticancer T cell activity. In mixed cultures of A2058 melanoma cells and PBMCs, treatment with CD3 agonist at sub-optimal doses triggered apoptosis in ~30% of cancer cells, whereas treatment with anti-PD-L1:TRAIL alone did not induce apoptosis (Fig. 3A). However, combination treatment with CD3 agonist and anti-PD-L1:TRAIL synergistically enhanced apoptosis in A2058 to over 60% (Fig. 3A). Further, co-treatment with anti-PD-L1:TRAIL also significantly increases IFN-γ secretion (Suppl. Fig. 1F). Correspondingly, in these mixed culture experiments the expression of PD-L1 on A2058 increased 3-fold (Suppl. Fig. 1G), whereas PD-1 expression on T cells increased 6-fold (Suppl. Fig. 1H). Importantly, treatment of activated T cells with PD-L1:TRAIL did not induce apoptosis in T cells (Suppl. Fig. 1I). Anti-PD-L1:TRAIL also significantly increased production of IFN-γ in mixed culture experiments with primary melanoma patient-derived TILs and autologous melanoma cells (Fig. 3B), to a level similar to that induced by anti-PD-L1 blocking antibody. PD-L1:TRAIL treatment of autologous primary cancer/TIL mixed culture experiments also increased apoptotic cell death in autologous cancer cells, both in melanoma and appendix carcinoma cells (Fig. 3C). The enhanced cytotoxicity of PBMCs upon CD3 agonist and anti-PD-L1:TRAIL treatment was blocked to medium levels by co-incubation with TRAIL-neutralizing antibody (2E5) (Fig. 3D) and was, therefore, dependent on TRAIL/TRAIL-receptor interaction. Correspondingly, anti-PD-L1:TRAIL induced apoptosis in mixed cultures of HCT-116 and PBMCs, but not in TRAIL resistant HCT-116.cFLIPs (Suppl. Fig. 1J), demonstrating that cell death upon anti-PD-L1:TRAIL treatment is TRAIL-mediated.

Of note, treatment with anti-EpCAM:TRAIL, PD-L1 blocking antibody or a combination did not significantly enhance the cytotoxic activity of PBMCs towards A2058 cells (Fig. 3A, D) nor did it increase the number of T cells (Fig. 3E). In these mixed cultures, the number of T-cells significantly increased upon anti-PD-L1:TRAIL treatment (Fig. 3E). Furthermore, in mixed cultures of A2058 and isolated $CD3^+$ T-cells, only anti-PD-L1:TRAIL treatment synergistically enhances apoptosis in A2058 to >80% (Fig. 3F) and significantly increases IFN-γ secretion (Fig. 3G). Thus, anti-PD-L1:TRAIL augments cytolytic activity of T cells in mixed culture experiments with T cells and tumor cells.

To mimic antigen-specific T cell activation, DLD-1 cancer cells were treated with T cell retargeting bispecific antibody (bsAb) anti-EpCAM:anti-CD3 (28). This bsAb retargets T cells to EpCAM-positive cancer cells and, in mixed cultures of DLD-1 and PBMCs, triggered cell death in an E:T ratio-dependent manner (Fig. 3H). Importantly, co-treatment with anti-EpCAM:anti-CD3 and anti-PD-L1:TRAIL significantly increased apoptotic elimination of DLD-1 cells leading to ~80% loss in cell viability after 24h of treatment at an E:T ratio of 5:1. (Fig. 3H). In contrast, control fusion protein anti-MCSP:TRAIL did not potentiate the cytotoxic effect of anti-EpCAM:anti-CD3 retargeted T cells (Fig.3H). In these mixed

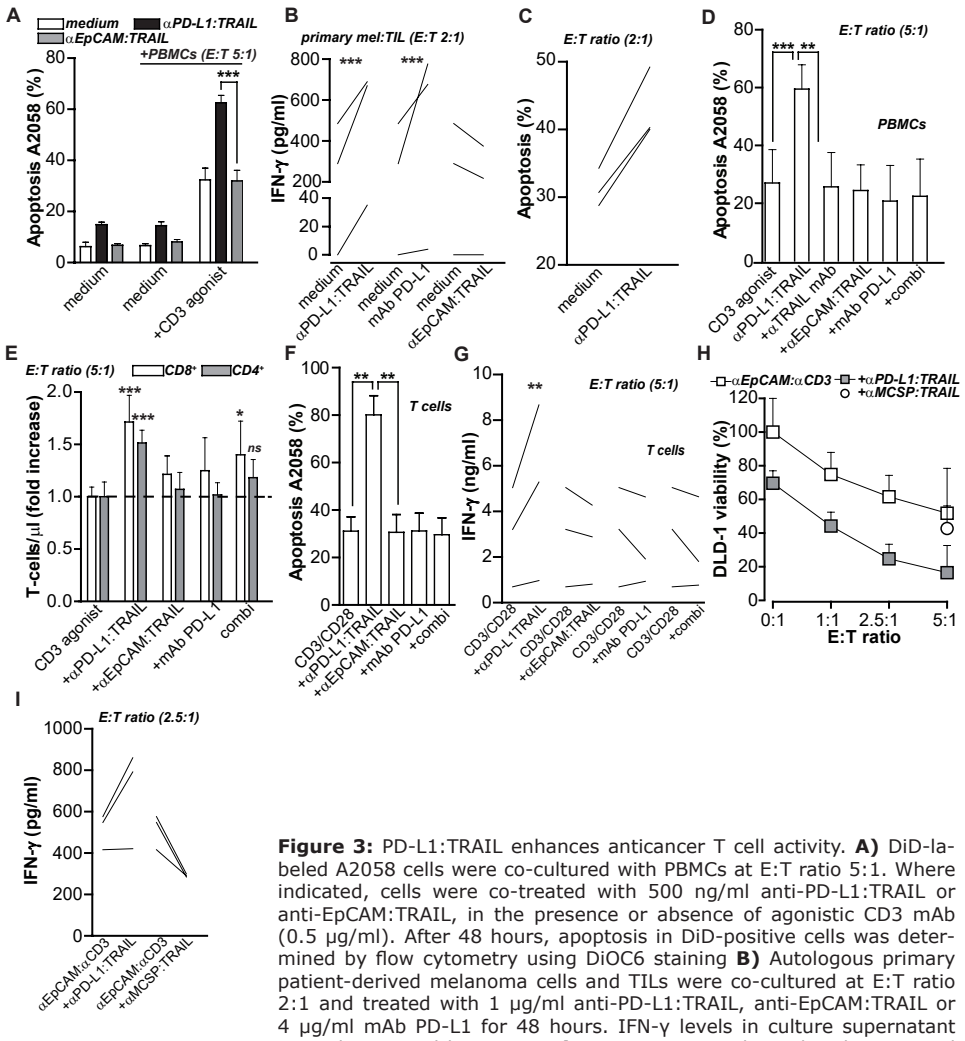

Figure 3: PD-L1:TRAIL enhances anticancer T cell activity. **A)** DiD-labeled A2058 cells were co-cultured with PBMCs at E:T ratio 5:1. Where indicated, cells were co-treated with 500 ng/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL, in the presence or absence of agonistic CD3 mAb (0.5 μg/ml). After 48 hours, apoptosis in DiD-positive cells was determined by flow cytometry using DiOC6 staining **B)** Autologous primary patient-derived melanoma cells and TILs were co-cultured at E:T ratio 2:1 and treated with 1 μg/ml anti-PD-L1:TRAIL, anti-EpCAM:TRAIL or 4 μg/ml mAb PD-L1 for 48 hours. IFN-γ levels in culture supernatant were determined by ELISA. **C)** Primary patient-derived melanoma and appendix carcinoma cells were co-cultured with autologous TILs at E:T ratio 2:1 and treated with 1 μg/ml anti-PD-L1:TRAIL for 48 hours, after which apoptosis was assessed by Annexin-V staining. **D)** DiD-labeled A2058 cells were co-cultured with PBMCs at E:T ratio 5:1 in the presence of agonistic CD3 mAb (0.5 μg/ml). Cells were co-treated with 500 ng/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL, where indicated cells were co-treated with TRAIL-neutralizing antibody (1 ug/ml). After 48 hours, apoptosis in DiD-positive cells was determined by flow cytometry using DiOC6 staining. **E)** In mixed cultures of PBMCs and A2058 as described in D, the PBMC population was stained with fluorescent CD4 and CD8 antibodies whereupon the number of $CD4^+$ and $CD8^+$ T cells within the PBMC gate was analyzed by flow cytometry. **F)** DiD-labeled A2058 cells were co-cultured with isolated $CD3^+$ T-cells at E:T ratio 5:1 in the presence of CD3/CD28 beads at a bead-to-cell ratio of 1:10. Cells were co-treated with 500 ng/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL and after 48 hours, apoptosis in DiD-positive cells was determined by flow cytometry using DiOC6 staining. **G)** IFN-γ levels in culture supernatant of F were determined by ELISA. **H)** DLD-1 cells were pre-seeded 24 hours before PBMCs were added at indicated E:T ratios in the presence of anti-EpCAM:anti-CD3 (50 ng/ml) with or without 500 ng/ml anti-PD-L1:TRAIL or anti-MCSP-TRAIL. Cell viability was determined by MTS after 24 hours. **I)** IFN-γ levels in culture supernatant of H were determined by ELISA. All graphs represent mean±SD. Statistical analysis was performed using two-way ANOVA (A), one-way ANOVA (D) or unpaired two-sided Student t test (B, F) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant).

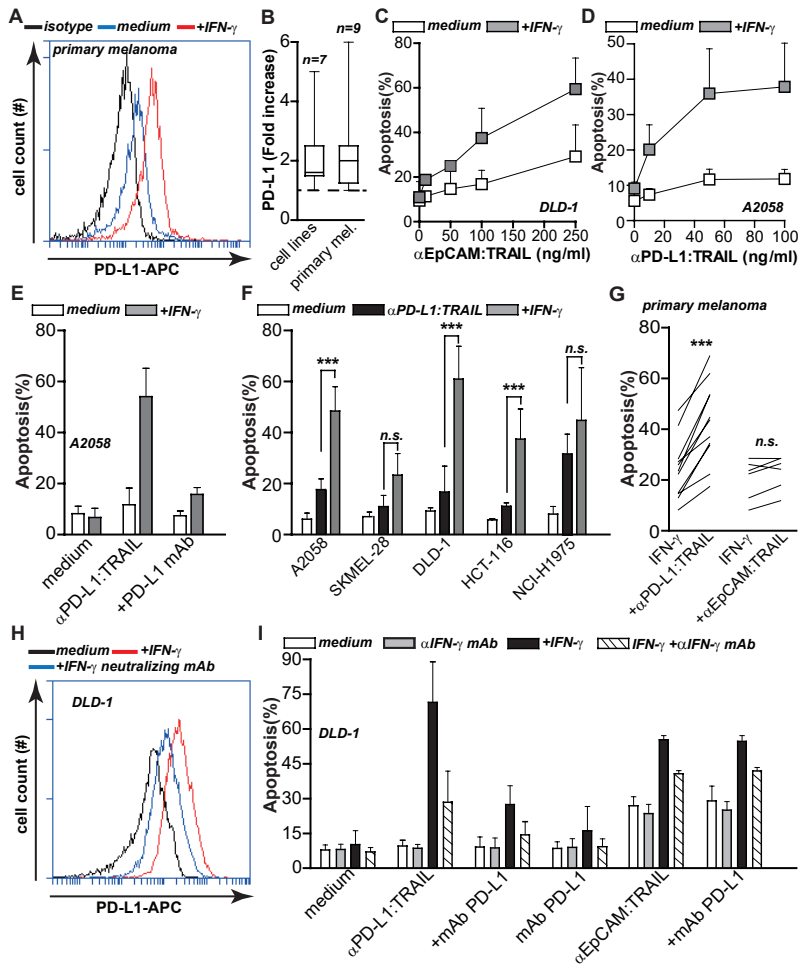

Figure 4: IFN-γ upregulates PD-L1 expression and sensitizes cancer cells to TRAIL-mediated apoptosis. **A)** Primary patient-derived melanoma cells were treated with or without 20 ng/ml IFN-γ for 24 hours after which PD-L1 expression was analyzed by flow cytometry. **B)** 7 cancer cell lines and 9 primary patient-derived melanoma cell cultures were treated with or without 20 ng/ml IFN-γ for 24 hours after which PD-L1 expression was analyzed by flow cytometry. Fold increase was calculated compared to non-treated cells. **C)** IFN-γ pre-treated or non-treated DLD-1 cells were incubated with an increasing dose of anti-EpCAM:TRAIL for 18 hours, after which apoptosis was assessed by flow cytometry using Annexin-V staining. **D)** IFN-γ pre-treated or non-treated A2058 cells were incubated with an increasing dose of anti-PD-L1:TRAIL. Apoptosis was assessed by Annexin-V staining after 18 hours. **E)** IFN-γ pre-treated or non-treated A2058 cells were incubated with 500 ng/ml anti-PD-L1:TRAIL in the presence or absence of PD-L1 blocking mAb (10 μg/ml). Apoptosis was determined by Annexin-V staining after 18 hours. **F)** A small panel of cancer cell lines were pre-treated with or without IFN-γ (20 ng/ml), followed by treatment of anti-PD-L1:TRAIL (500 ng/ml) for additional 18 hours. Apoptosis was determined by Annexin-V staining. **G)** IFN-γ pre-treated primary patient-derived melanoma cultures were treated with 1 μg/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL for 48 hours. Apoptosis was determined using Annexin-V. **H)** DLD-1 cells were treated with or without 20 ng/ml IFN-γ in the presence or absence of 8 μg/ml IFN-γ neutralizing mAb. After 24 hours, PD-L1 expression was analyzed by flow cytometry. **I)** DLD-1 cells were pre-treated with or without 20 ng/ml IFN-γ in the presence or absence of 8 μg/ml IFN-γ neutralizing mAb. After 24 hours, cells were treated with anti-PD-L1:TRAIL (250 ng/ml) in the presence or absence of PD-L1 blocking mAb (10 μg/ml), anti-EpCAM:TRAIL (250 ng/ml) or mAb PD-L1 (1 μg/ml). All graphs represent mean±SD. Statistical analysis was performed using two-way ANOVA (F) or Wilcoxon matched pairs test (G) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant).

cultures, IFN-γ production increased upon co-treatment with anti-EpCAM:anti-CD3 and anti-PD-L1:TRAIL when compared to co-treatment with anti-MCSP:TRAIL (Fig. 3I).

IFN-γ enhances PD-L1 expression and sensitizes cancer cells to TRAIL-mediated apoptosis

IFN-γ up-regulates PD-L1 expression on cancer cells,[11] a finding confirmed here in a panel of 7 cancer cell lines and 9 primary patient-derived melanoma cell cultures (Fig. 4A, B). Since pro-apoptotic activity of anti-PD-L1:TRAIL is dependent on PD-L1 specific binding to cells, this upregulation of PD-L1 may sensitize cancer cells to anti-PD-L1:TRAIL-mediated killing. Further, IFN-γ is known to sensitize cancer cells to TRAIL-mediated apoptosis, e.g. through down-regulation of cFLIP.[30, 31] Correspondingly, apoptotic activity of control fusion protein anti-EpCAM:TRAIL on DLD-1 cells was increased by IFN-γ pre-treatment (Fig. 4C). This effect was due to TRAIL sensitization and not due to EpCAM upregulation as IFN-γ does not affect EpCAM expression.[32] Thus, IFN-γ both upregulates PD-L1 and sensitizes cancer cells to TRAIL, which may augment anti-PD-L1:TRAIL activity. In line with the above, pre-treatment of A2058 cells with IFN-γ dose-dependently sensitized A2058 cells to anti-PD-L1:TRAIL-mediated apoptosis (Fig. 4D), as well as a further panel of 5 cancer cell lines (Fig. 4F) and 11 primary melanoma cell cultures (Fig. 4G). This apoptotic activity of anti-PD-L1:TRAIL was still abrogated by co-treatment with excess PD-L1 blocking antibody (Fig. 4E). Additionally, co-treatment with IFN-γ neutralizing antibody inhibited IFN-γ mediated PD-L1 upregulation on DLD-1 cells (Fig. 4H) and abrogated anti-PD-L1:TRAIL-mediated apoptosis (Fig. 4I). Thus, the apoptotic activity of anti-PD-L1:TRAIL is enhanced by IFN-γ, likely due to both upregulation of PD-L1 expression on cancer cells and simultaneous sensitization of cancer cells to TRAIL-mediated apoptotic signaling.

Anti-PD-L1:TRAIL converts PD-L1 expressing myeloid cells into pro-apoptotic tumoricidal effector cells

Within the tumor micro-environment, various types of infiltrated myeloid cells, such as M2 macrophages and DCs, are known to express PD-L1 and to suppress antitumor immunity.[33, 34] On these cells, PD-L1 expression is further elevated by tumor localized IFN-γ.[4, 5] Previously, we demonstrated that direct arming of myeloid effector cells using a TRAIL fusion protein that binds to CLL-1 on granulocytes, enhanced the pro-apoptotic activity of such myeloid cells.[26] To assess whether potentiation of myeloid effector cell activity might also contribute to anti-PD-L1:TRAIL activity, we generated various myeloid effector cell populations, i.e. monocytes, M0, M1 and M2 macrophages, immature DCs (iDCs) and mature DCs (mDCs). All of these effector cells expressed PD-L1 to a varying degree, with monocytes having lowest and mDCs having the highest expression (Fig. 5A). Further, M1 macrophages had higher PD-L1 expression compared to the immunosuppressive M2 macrophages, a finding in line with an earlier report.[29] PD-L1 expression was upregulated by IFN-γ pre-treatment, as illustrated for monocytes (Fig. 5B). In subsequent mixed culture experiments of monocytes and DLD-1 target cells, such IFN-γ pre-treated monocytes alone did not significantly induce apoptosis in DLD1

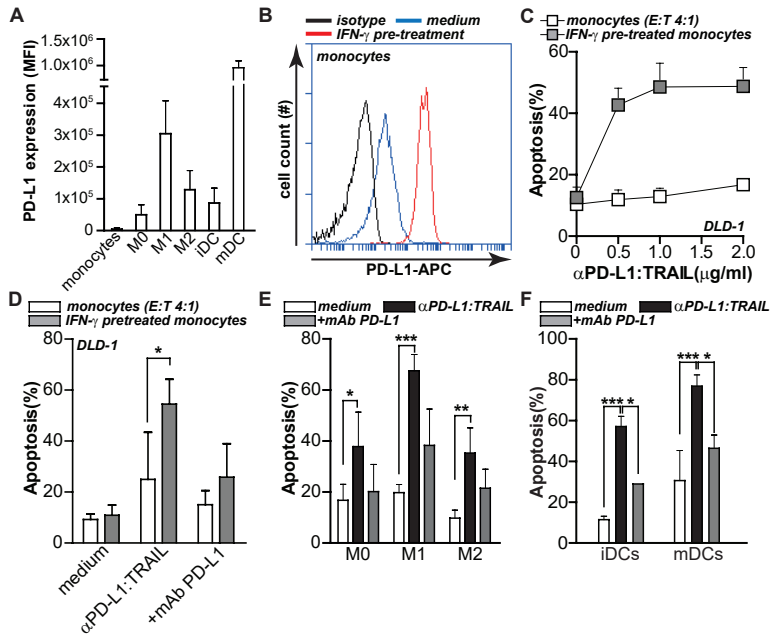

Figure 5: Anti-PD-L1:TRAIL converts PD-L1 expressing myeloid cells into pro-apoptotic tumoricidal effector cells. **A)** PD-L1 expression levels of monocytes, M0, M1, M2 macrophages, immature and mature DCs were determined by flow cytometry. Isotype control MFI was subtracted from original MFI. **B)** Monocytes were treated with or without 20 ng/ml IFN-γ for 24h after which PD-L1 expression was analyzed by flow cytometry. **C)** Monocytes were pre-treated with or without 20 ng/ml IFN-γ for 24 hours, washed twice with PBS after which DLD-1 cells were added at E:T ratio 4:1 in the presence of an increasing dose of anti-PD-L1:TRAIL. After 18 hours, apoptosis was assessed by Annexin-V staining. **D)** As in C with 500 ng/ml anti-PD-L1:TRAIL with or without PD-L1 blocking mAb (10 μg/ml). **E)** M0, M1 or M2 macrophages were co-cultured with DLD-1 cells at E:T ratio 4:1 in the presence of 500 ng/ml anti-PD-L1:TRAIL with or without PD-L1 blocking mAb (10 μg/ml). After 18 hours, apoptosis was assessed by Annexin-V staining. **F)** Immature or mature DCs were co-cultured with DLD-1 cells at E:T ratio 4:1 in the presence of 500 ng/ml anti-PD-L1:TRAIL with or without PD-L1 blocking mAb (10 μg/ml). After 18 hours, apoptosis was assessed by Annexin-V staining. All graphs represent mean±SD. Statistical analysis was performed using two-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant).

cells (Fig. 5C). However, treatment with anti-PD-L1:TRAIL dose-dependently increased apoptosis in DLD-1 cells (Fig. 5C). This increase was blocked by co-treatment with molar excess of PD-L1 blocking mAb (Fig. 5D). Similarly, M0, M1 or M2 macrophages and immature or mature DCs alone minimally induced apoptosis of DLD-1 cells in mixed culture experiments (Fig. 5E, F). However, addition of anti-PD-L1:TRAIL to these mixed cultured significantly triggered apoptosis in DLD-1 cells (Fig. 5E, F), with e.g. an >50% increase in apoptosis in DLD-1 cells in mDC mixed culture experiments (Fig. 5F). In all mixed culture experiments, PD-L1 blocking mAb strongly inhibited the apoptotic activity of anti-PD-L1:TRAIL (Fig. 5D-F). Of note, background apoptosis in DLD-1 cells was reduced from ~20 to ~10% in the presence of M2 macrophages, a finding in line with their pro-tumorigenic role (Fig. 5E). Further, although M1 macrophages express higher levels of PD-L1 (Fig. 5A) and were intrinsically more cytolytic (Fig. 5E), the potentiating effect of anti-PD-L1:TRAIL was most pronounced for M2 macrophages, with a 4-fold increase in apoptosis with M2 vs. a 3-fold increase with M1 macrophages (Suppl. Fig. 1K).

Taken together, these data demonstrate that anti-PD-L1:TRAIL binds to PD-L1 on myeloid cells, whereupon these normally immunosuppressive cells trigger TRAIL-mediated apoptotic cell death in cancer cells.

Discussion

Here, we describe a new PD-L1/PD-1 checkpoint inhibitor approach that combines PD-L1 checkpoint blockade with targeted delivery of the pro-apoptotic tumoricidal protein TRAIL. Fusion protein anti-PD-L1:TRAIL has a multi-fold therapeutic effect, depicted in Fig. 6, comprised of 1. induction of TRAIL-mediated cancer cell death after binding to tumor-expressed PD-L1, 2. reactivation of antitumor T-cells by blocking of PD-L1/PD-1 interaction, 3. converting suppressive monocytes/macrophages/DCs into pro-apoptotic effector cells that trigger TRAIL-mediated cancer cell death and 4. enhancement of IFN-γ production by immune effector cells, leading to simultaneous PD-L1 upregulation and sensitization of cancer cells to TRAIL.

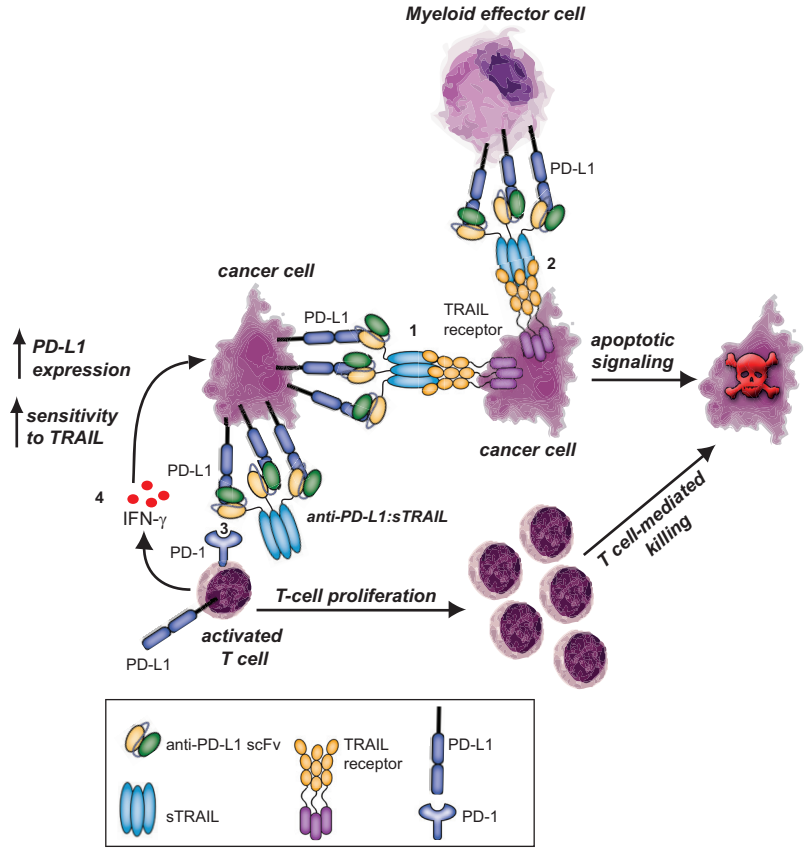

Figure 6: Proposed mechanism of action for anti-PD-L1:TRAIL. anti-PD-L1:TRAIL induces TRAIL-mediated cancer cell death after binding to tumor-expressed PD-L1 **(1)** or after binding to PD-L1 on myeloid effector cells **(2)**, restores proliferation and antitumor activity of T cells by blocking PD-L1/PD-1 interaction **(3)** and enhances IFN-γ production of T cells, leading to simultaneous PD-L1 upregulation and sensitization of cancer cells to TRAIL **(4)**.

Blockade of the PD-L1/PD-1 axis by anti-PD-L1:TRAIL enhanced T cell activation, proliferation and IFN-γ production, an effect similar to that reported for anti-PD-L1 monoclonal antibodies,[35] and triggered TRAIL-mediated apoptosis in cancer cells. TRAIL has strong pro-apoptotic activity towards various cancers in the absence of deleterious activity towards normal cells.[36] Clinical evaluation of first-generation recombinant human TRAIL (rhTRAIL, brand name "dulanermin") and TRAIL receptor antibodies typically yielded low to absent toxicity towards normal cells and tissues (reviewed in[37]), with a maximum tolerable dose (MTD) not being reached for dulanermin.[38] Of note, dulanermin did not have toxicity towards immune cells in these studies.[37, 38] Thus, the use of TRAIL as additional effector domain is anticipated to have no or only minimal side-effects when combined with PD-L1 blockade strategies. In line with this, we did not detect any negative effects of the TRAIL domain of PD-L1:TRAIL on T cells, with no increase in apoptosis in activated T cells. A recent study did report suppression in T cell activation and proliferation when T-cells were co-stimulated with recombinant TRAIL and CD3/CD28 antibodies.[39] However, this suppressive effect was only observed at a concentration of recombinant TRAIL of 50 µg/ml, which is ~50 fold higher than the highest concentration of 1 ug/ml typically used in literature and by us here for PD-L1:TRAIL.

Of note, early clinical trials in various types of malignancies yielded only limited clinical benefit for dulanermin with stable disease being the best-reported activity.[38, 40, 41] However, it has become evident that first generation TRAIL receptor-agonists do not optimally exploit the unique apoptotic signaling characteristics of the various TRAIL receptors (reviewed in[42-44]). Most notably, apoptotic signaling via TRAIL-R2, one of the two agonistic TRAIL receptors, is not efficiently achieved by soluble homotrimeric rhTRAIL, as TRAIL-R2 requires binding of membrane-bound TRAIL or secondarily cross-linked rhTRAIL.[21, 45, 46] Since TRAIL-R2 is often highly expressed on cancer cells it forms an important target for TRAIL-based therapy. Importantly, we and others have previously shown that tumor-directed scFv:TRAIL fusion proteins effectively activate TRAIL-R2 on targeted cancer cells only. In this process high affinity and tumor-selective binding via its scFv domain converts a soluble and essentially inactive scFv:TRAIL fusion protein into membrane-bound TRAIL with highly potent agonistic activity towards both TRAIL-R1 and TRAIL-R2.[21, 47, 48] Thus, anti-PD-L1:TRAIL has combined and mutuallyreinforcing PD-L1-blocking and TRAILR agonistic activities within one therapeutic anticancer fusionprotein, which outperforms combined treatment with PD-L1-blocking antibody and TRAIL.

Importantly, in mixed culture experiments using primary patient-derived melanoma cells and autologous TILs, treatment with anti-PD-L1:TRAIL enhanced IFN-γ production and augmented TIL-mediated cancer cell apoptosis. Additionally, when T cells were redirected to carcinoma cells using an anti-EpCAM:anti-CD3 bispecific antibody, anti-PD-L1:TRAIL synergistically enhanced their pro-apoptotic antitumor activity. A similar potentiating effect of PD-L1 blockade has been previously reported for T cell retargeting BiTEs that target CEA or CD33.[49, 50] This suggests that anti-PD-L1:TRAIL may be exploited to augment tumor-specific activity of T cells. In line with this, anti-PD-L1:TRAIL also increased IFN-γ secretion in a model antigen-specific reaction where T-cells from CMV-positive donors were stimulated with CMV protein pp65.

Tumor cells that do not constitutively express PD-L1 can rapidly upregulate PD-L1 when the tumor micro-environment is infiltrated by T cells. As previously reported, this up-regulation is likely due to IFN-γ generated by T cells upon tumor cell recognition and attempted elimination.[12] In the context of anti-PD-L1:TRAIL, this IFN-γ production may contribute to its antitumor efficacy as IFN-γ not only sensitizes cancer cells to TRAIL but also upregulates PD-L1 expression.[11, 30, 31] Thus, anti-PD-L1:TRAIL may trigger a feed-forward loop of increasing IFN-γ, increasing PD-L1 expression and increasing TRAIL sensitivity.

In the tumor microenvironment, various myeloid cells such as macrophages and DCs, also express PD-L1 and hereby can suppress antitumor immunity.[33, 34] The presence of these suppressive cell types correlated with disease progression and reduced survival in HCC and breast cancer patients.[6, 51] Here, we showed that anti-PD-L1:TRAIL can arm PD-L1-expressing monocytes, DCs and macrophages with TRAIL, turning these suppressive cell types into pro-apoptotic effector cells whilst simultaneously blocking potential PD-L1-mediated immunosuppressive effects. The obvious promise of this arming strategy is illustrated by reports where PD-L1 blockade alone significantly improved the *in vivo* antitumor activity of T cells treated with suppressive DCs or monocytes.[6, 33]

Interestingly, based on the known receptor interactions of PD-1 ligands (reviewed in[3]), PD-1 antibodies may have distinct biological activities from PD-L1 antibodies and their activities may not be redundant, depending on the dominant interaction for a particular cancer. In this respect, simultaneous blockade of PD-1 and PD-L1 maximized cytolytic T cell activity of tumor-directed bispecific T cell engaging antibodies.[49]. These findings suggest that combining anti-PD-L1:TRAIL with PD-1 blocking antibodies may further optimize checkpoint inhibitor-based therapy.

In conclusion, fusion protein anti-PD-L1:TRAIL has promising multi-fold and mutually reinforcing therapeutic effects comprised of PD-L1 checkpoint blockade and simultaneous induction of TRAIL-mediated apoptosis. This new fusion protein may provide possibilities to enhance the efficacy of therapeutic PD-L1/PD-1 checkpoint inhibition alone or in combination with other immunotherapeutic strategies.

Acknowledgements

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Conflict of interest

The authors state no conflict of interest.

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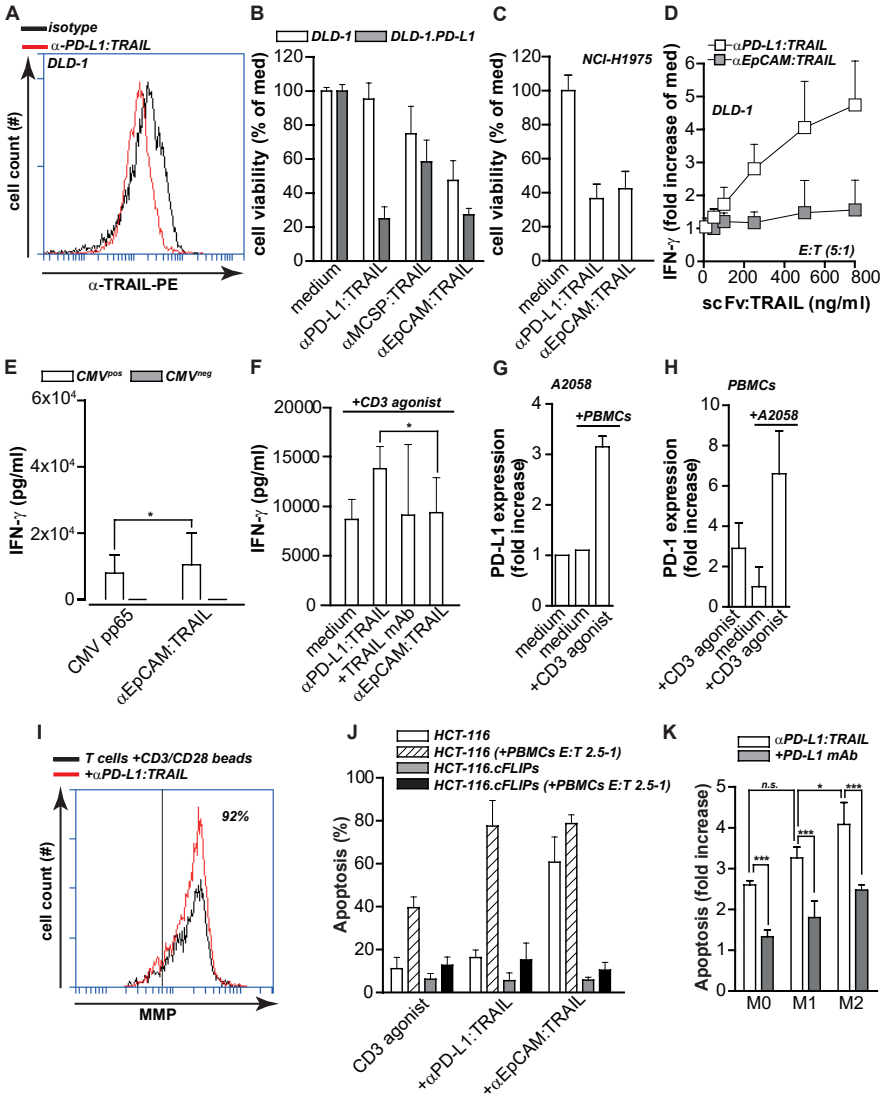

Supplementary Figure 1: A) Binding of anti-PD-L1:TRAIL to DLD-1 cells was analyzed by flow cytometry. **B)** Spheroid formation of DLD-1.PD-L1 or DLD-1 cells was assessed in the presence or absence of 100 ng/ml anti-PD-L1:TRAIL, anti- MCSP:TRAIL or anti-Ep-CAM:TRAIL. Cell viability was determined by MTS after 72 hours. **C)** Spheroid formation of NCI-H1975 cells was assessed in the presence or absence of 1 µg/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL. Cell viability was determined by MTS after 72 hours. **D)** DLD-1 cells were co-cultured with PBMCs at E:T ratio 5:1 in the presence of agonistic CD3 mAb (0.5 µg/ml) and an increasing dose of anti-PD-L1:TRAIL or anti-EpCAM:TRAIL. After 72 hours, IFN-γ levels in culture supernatant were determined by ELISA. **E)** PBMCs from CMV-positive or CMV-negative donors were treated with 500 ng/ml anti-EpCAM:TRAIL in the presence of CMV protein pp65 for 96 hours. IFN-γ levels in culture supernatant were determined by ELISA. **F)** A2058 cells were co-cultured with PBMCs at E:T ratio 5:1 and treated with 500 ng/ml anti-PD-L1:TRAIL or anti-EpCAM:TRAIL as indicated, in the presence of agonistic CD3 mAb (0.5 µg/ml). Where indicated, cells were co-treated with TRAIL-neutralizing antibody (1 ug/ml). After 48 hours,

IFN-γ levels in culture supernatant were determined by ELISA. **G)** A2058 cells were co-cultured with PBMCs at E:T ratio 5:1 in the presence or abscence of agonistic CD3 mAb (0.5 µg/ml).After 48 hours, PD-L1 expression on A2058 cells was measured by flow cytometry. **H)** As in G, PD-1 expression on PBMCs was measured by flow cytometry. Fold increase was calculated compared to medium control. **I)** A2058 cells were co-cultured with isolated $CD3^+$ cells at E:T ratio 5:1. Cells were co-treated with 500 ng/ml anti-PD-L1:TRAIL in the presence of CD3/CD28 beads. After 48 hours, loss of mitochondrial membrane potential (MMP) in T cells was measured by flow cytometry. **J)** As in F, HCT-116.wt or TRAIL resistantHCT-116.cFLIPs cells were co-cultured with PBMCs at E:T ratio 2.5:1 and loss of mitochondrial potential was assesed after 48 hours. **K)** M0, M1 or M2 macrophages were co-cultured with DLD-1 cells at E:T ratio 4:1 in the presence of 500 ng/ml anti-PD-L1:TRAIL with or without PD-L1 blocking mAb (10 µg/ml). After 18 hours, apoptosis was assessed by Annexin-V staining. Fold increase was calculatedcompared to medium control. All graphs represent mean ±SD. Statistical analysis was performed using Wilcoxon matched pairs test (E), unpaired two-sided Student t test (F) or ANOVA (K). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant)

cell line / patient sample	relative expression level	fold increase with IFN-γ
DLD-1	+	5
DLD-1.PD-L1	++++	2
A2058	+	1.5
HCT-116	+	2.5
SKMEL-28	++	1.5
MDA-MB-231	++++	nd
ES-2	++	nd
NCI-H1975	++	nd
Melanoma 1	+++	6
Melanoma 2	+++	1
Melanoma 3	++++	2
Melanoma 4	++	1.5
Melanoma 5	+	2
Melanoma 6	+++	2
Melanoma 7	+	2
Melanoma 8	+	3
Melanoma 9	+	1
Melanoma 10	+	4
Appendix Carcinoma	++++	2

Supplementary Table 1: PD-L1 expression levels of all cell lines and primary samples used in this study were determined by flow cytometry using PD-L1-APC antibody with appropriate isotype control. Relative expression levels were calculated by subtracting isotype control MFI from original MFI. (Relative expression index: MFI <20.000 = +, 20.000 <MFI> 50.000 = ++, 50.00 <MFI> 100.000 = +++, MFI >100.000 = ++++). Cells were also pre-treated with or with-out 20 ng/ml IFN-γ for 24 hours, after which PD-L1 expression was determined using flow cytometry. Fold increase was calculated compared to non-treated cells (nd=not determined).